Studies on Toll–pathway genes of tiger shrimp, *Penaeus monodon*

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1. INTRODUCTION

Crustaceans, dominated by shrimp, represent one of the most economically important sectors of the rapidly growing aquaculture food industry. Shrimp aquaculture industry is subjected to significant economic losses due to susceptibility to a wide variety of pathogens including viruses, bacteria and parasites (Bachere, 2000; Flegel, 2006; Hauton, 2012). Consequently, developing effective strategies to control and prevent diseases is a priority for economically sustainable aquaculture. In this, crustacean immunology, especially of shrimp, is a significant area of research owing to the key importance of farmed shrimp as the most valuable food commodity and due to the unique immune system shrimp possesses compared to the vertebrates.

For the prevention of diseases, it is crucial to develop quantitative assays in order to evaluate and monitor the immune state of the animal (Bachere, 2000). Crustaceans depend on innate defence system for evading pathogens as they have no adaptive memory; in other words, they do not have the ability of producing immunoglobulins (Roch, 1999). Understanding shrimp defence mechanisms in combination with different strategies can contribute to improve disease management. Several different methods exist to understand the health status of these animals, including the evaluation of physiological parameters such as haemolymph protein concentration, total haemocyte counts, phenoloxidase activity, free–radical production, phagocytic activity, and field variables including stress tests, survival, and growth rates, which can be used as shrimp health status indicators (Rodríguez and Moullec, 2000). Many immune–related molecules in penaeid shrimp have been discovered, using the analysis of expressed sequence tag libraries, microarray studies and proteomic approaches. These include antimicrobial peptides, serine proteases and inhibitors, phenoloxidase, oxidative enzymes, clotting proteins, pattern recognition proteins, lectins, Toll receptors, and other humoral factors that might participate in the innate immune system of shrimp (Tassanakajon et al., 2013).

The innate defence system – also known as natural or non–specific defense system –includes both cellular and humoral components with a considerable integration of both the components to produce a single coordinated system for the detection and elimination of foreign organisms potentially hazardous for the host (Jiravanichpaisal et al., 2006). The cuticle functions as the first physical barrier, and it contains antimicrobial substances (Soderhall and Cerenius,

1992). Once the pathogen has crossed the outer defense barriers, haemocytes play an important role in the crustacean immune response. In addition to participating in the inactivation of invading organisms, haemocytes are also involved in the regulation of different physiological functions i.e., exoskeleton hardening, cuticle damage healing, coagulation, carbohydrate metabolism, and protein/amino acid transportation and storage (Jiravanichpaisal et al., 2006). Cellular defense components include all those reactions performed directly by haemocytes (phagocytosis, encapsulation, nodule formation). On the other hand, the humoral components include the activation and release of molecules stored within haemocytes, such as anticoagulant proteins, agglutinins (lectins), phenoloxidase enzyme, antimicrobial peptides, protease inhibitors, etc (Holmblad and Soderhall, 1999). The traditional strategy of vaccination used to prevent viral diseases in vertebrates is not considered effective to cure viral disease of shrimp, since no adaptive immunity exists in them. However, experimental data demonstrate specific memory phenomena in invertebrates (Rowley and Pope, 2012).

The Indian aquaculture industry has flourished over the last three decades. The marine tiger shrimp *Penaeus monodon* is one of the important and indigenous farmed shrimp species. Diseases affecting shrimp have caused severe losses to the aquaculture industry and led to a steep decline in culture of *P. monodon*, which was one of the most widely farmed shrimp species all over the world, before the exotic species *Penaeus (Litopenaeus) vannamei* has been introduced and farmed widely. In order to generate protection strategies for sustainable shrimp culture, it is essential to gain better understanding of the defense system of shrimp. The identification of immune genes and the knowledge of the expression patterns of these genes in the presence and absence of pathogens will provide much needed information on the role of the immune genes in the shrimp immune system. In this background, a study has been envisaged with the following objectives.

1. To identify and characterise different Toll–pathway genes in *Penaeus monodon*
2. To study the ontogenetic and tissue–level expression of Toll–pathway genes of *P. monodon*
3. To study the *in–vivo* modulation of expression of selected Toll–pathway genes in response to WSSV and *Vibrio harveyi* infection
4. To study the expression of Toll–pathway genes *in vitro* in response to WSSV and selected pathogen–mimicking ligands using primary culture of haemocytes of *P. monodon*

2. REVIEW OF LITERATURE

2.1. Immune mechanisms in shrimps

In crustaceans, many physical/anatomical barriers such as cuticular exoskeleton covering the body and chitinous lining of the intestinal epithelium, known as peritrophic membrane, protect the animal from direct access to pathogens entering through body surface and oral route. However, when these barriers are breached by pathogens, an efficient immune system plays the key role in dealing with pathogen invasion and thereby combating disease conditions. Immune mechanism helps in resisting pathogen invasion through recognition of non–self–molecules followed by the mobilisation of various cells and molecules to initiate neutralisation of the invading pathogens. The immune responses have been categorised into innate (natural) and adaptive (acquired) and it is recognised that while invertebrates possess predominantly innate immune (non–specific) responses, adaptive (specific) immunity is dominant in higher vertebrates. Innate immune system is evolutionarily conserved from plants to humans and is the first line of defense in all animals, known to be involved in the recognition of non–self–particles and controlling infection in its early stages (Vazquez et al., 2009). Invertebrates such as shrimp depend mostly on innate immune factors as they lack a true adaptive immune response. Innate immune responses are brought about by effector mechanisms that lead to elimination of pathogens by inducing oxidative modifications using the proPO system, through proteolytic processes and through the process of phagocytosis which can be brought about either by opsonisation or specific binding with cellular receptors which in turn facilitates phagocytosis of the infectious foreign particles by haemocytes (Medzhitov and Janeway, 1997; Caroll and Prodeus, 1998). Although they possess less complex immune system compared to higher vertebrates, invertebrates which exist in diverse habitats are able to defend themselves from invading pathogens through potent innate immune response, (van de Braak 2002; Jiravanichpaisal et al. 2006).

The innate immune system has two components–cellular and humoral, and these together make up an efficient immune system in shrimp as well as other crustaceans to evade the pathogens. The cellular responses are carried out by haemocytes present in haemolymph of shrimp and include phagocytosis, encapsulation, nodule formation, and apoptosis (Xu et al.,

2014). The important humoral responses in shrimp include the prophenoloxidase (proPO) system along with melanisation, the clotting cascade, pathogen recognition mediated through different pathogen recognition receptor proteins (PRRs) like Dscam, lectins Toll, IMD and generation of a wide array of antimicrobial peptides (Musthaq and Kwang, 2014, Tassanakajon et al., 2018)

2.2. Cellular immune responses

Haemocytes (invertebrate blood cells) of shrimp play a crucial role in host defence against pathogens as they are the main mediators of cellular immune responses and form the first line of defense once the pathogens overcome the physical barriers. These barriers include rigid cuticular exoskeleton with antimicrobial substances, gut enzymes and acids that can digest the pathogens and the chitinous peritrophic membrane lining the intestinal epithelium. Moreover, haemocytes are the effectors of cellular immune responses as well as source of effectors for humoral immune responses. In shrimp three different types of haemocytes have been classified namely granular, semigranular and hyaline cells (Sung et al., 1999; Johansson et al., 2000). The different haemocyte types have specific or partially overlapping functions wherein the granular cells have been observed to function majorly in the prophenoloxidase (proPO) system and cell–mediated cytotoxic response whereas hyaline cells are found to be involved in phagocytosis, and the semigranular cells contribute to the proPO system, as well as encapsulation, phagocytosis, and cytotoxic response (Cerenius et al., 2008; Xu et al., 2014; Tassanakajon et al., 2018). The effector molecules involved in the cellular and humoral responses are released through the activation of several signalling pathways (Tassanakajon et al., 2013), and the activation is initiated by the recognition of the invariant molecular signatures associated with the pathogens known as pathogen associated molecular patterns (PAMPs) by pattern recognition proteins (PRPs).

Phagocytosis, is the internalisation of small foreign particles by individual cells and is considered to be the major cellular defence mechanism in crustaceans. Upon internalisation of the foreign particles, shrimp haemocytes, use cytotoxic oxygen radicals to eliminate the foreign particles (Song and Hsieh, 1994; Munoz et al., 2000). Among the different haemocytes, the primary cells involved in the phagocytosis of foreign particles in shrimp are the semigranular haemocytes (Soderhall and Cerenius, 1992; Bodhipaksha and Weeks–Perkins, 1994; Bachere et al., 1995). Apart from the semigranular haemocytes, the granular haemocytes can also carry out

the process of phagocytosis of foreign matter while the smallest haemocytes–the hyaline cells are also considered as phagocytes (Hose and Martin, 1989; Soderhall and Cerenius, 1992). The process of phagocytosis consists of chemotaxis, adherence, ingestion, pathogen destruction and exocytosis (Kondo et al., 1998; Vargas–Albores and Yepiz–Plascencia 1998). Elimination of the phagocytosed foreign particles occurs via the epithelium of the gills, gut, hepatopancreas and extremities of periopod and pleopods. Encapsulation and nodulation are cellular responses that are limited to invertebrates in defence against large foreign particles which cannot be phagocytosed by individual cells and form an important defense mechanism in shrimps. When the size of the foreign bodies is too large to be phagocytosed or the particles entering the body are in large numbers, several haemocytes combine together to seal off the invaders (Soderhall and Cerenius 1992; Soderhall et al., 1996). Unlike phagocytosis, encapsulation results in a multilayered, overlapping sheath, of haemocytes around the invaders while in nodule formation, invading micro–organisms become enmeshed in a central core of melanised haemocytes surrounded by a wall of flattened haemocytes, leading to their isolation from the rest of the host (Ratcliffe et al., 1985; Johansson and Soderhall, 1989). Semi–granulocytes are capable of recognising the invading foreign agents and bring about their encapsulation with opsonin–like proteins (peroxinectin) associated with the proPhenoloxidase (proPO) activation system and besides being an encapsulation promoter these proteins also act as a degranulation and adhesion factor for the semi–granulocytes and granulocytes, (Vargas–Albores and Yepiz–Plascencia, 1998; Wang et al., 2001; van de Braak, 2002). In nodule formation a large number of haemocytes participate synergistically to trap microorganisms or large antigens and form haemolytic nodules that can be detected in gill and hepatopancreas (Aguirre–Guzman et al., 2009). Finally, the destruction of micro–organisms is brought about by activation of the proPO system and melanisation (Wang et al., 2001; van de Braak, 2002).

Apoptosis also known as programmed cell death is a crucial process for eliminating infected cells, thus preventing the spread of infection and is an important cellular immune response in shrimps. There have been many reports stating the importance of apoptosis in shrimp immunity and evidences suggesting induction of apoptosis upon virus infection (Zhi et al., 2011; Leu et al., 2013; Zuo et al., 2011; Xu et al., 2014). Several apoptosis–related genes (caspases, inhibitor of apoptosis protein (IAP), apoptosis inducing factor (AIF), cytochrome c, Fortilin or translationally controlled tumor protein (TCTP), apoptosis signal–regulating kinase 1 (ASK1) etc.

have been identified from different groups of crustaceans. In shrimp, genes regulating apoptosis have been identified including several different caspases from different species such as *P. monodon*, *P. vannamei, M. japonicus Penaeus* (*Fenneropenaeus*) *merguiensis* (Wongprasert et al., 2007; Wang et al., 2008; Leu et al., 2013; Wang et al., 2013b).

2.3. Humoral immune responses

In crustaceans, soluble factors secreted by granulocytes, such as lectins, agglutinins, antimicrobial peptides (AMPs), proPO etc. constitute the effectors of humoral immunity. As in the case of invertebrates, the humoral responses form the first line of defence against pathogens in shrimp and hence form a crucial part of the shrimp defence system (Tassanakajon et al., 2018). Pathogen–associated molecular patterns (PAMPs) or the viral protein antigens, upon detection by the receptors on the cell–surface or pattern recognition receptors (PRRs) lead to humoral responses including the activation of NF–kB signalling pathways (Li and Xiang, 2013; Tassanakajon et al., 2018).

2.3.1. Prophenoloxidase system

A diverse array of humoral responses have been studied in invertebrates and these include the clotting cascade, anti–oxidant defense enzymes like superoxide dismutase, peroxidase, catalase and nitric oxide synthase, defensive enzymes like lysozyme, acid phosphatase and alkaline phosphatase, reactive oxygen and nitrogen intermediates and antimicrobial peptides, however, one of the most effective and most widely studied humoral immune responses is the cellular melanotic encapsulation. The process of melanisation employs circulating haemocytes and several proteins associated with the prophenoloxidase (proPO)–activating system and is a major innate immune response in shrimp (Amparyup et al., 2013; Tassanakajon et al., 2018). The proPO cascade is triggered upon recognition of the microbial–derived PAMPs and activation of the serine proteinases cascade by the PAMPs–PRP interactions. The active enzyme of this cascade – phenoloxidase is generated upon proteolysis of the proPO precursor by serine proteases and the terminal serine proteinase is called proPO activating factor (PPA) or proPO activating enzymes (PPAEs) (Sritunyalucksana and Soderhall, 2000; Tassanakajon et al., 2018). The proPO gene was first cloned from *P. monodon* (Sritunyalucksana et al., 1999). Subsequently in shrimps

several proPO system– or melanisation– related genes have been identified and their role in shrimp immunity has been determined (Tassanakajon et al., 2018).

2.3.2. Clotting cascade

Coagulation of haemolymph is an important innate immune mechanism as it prevents the loss of haemolymph as well as invasion of pathogens upon injury and is brought about by clottable proteins. These proteins act as important defence molecules having multifunctional properties and are known to be involved in the recognition of non–self particles and their subsequent neutralisation (Iwanaga and Lee, 2005, Tassanakajon et al., 2018). Haemolymph clotting in crustaceans is brought about by polymerisation of clottable proteins (CPs) present in plasma, catalysed by calcium ion – dependent transglutaminases (TGase) that are released from haemocyte upon injury (Sritunyalucksana and Soderhall, 2000, Lee and Soderhall, 2002). The CPs and TGases have been identified in important shrimp species like *P. monodon* (Yeh et al., 1999; Chen et al., 2005; Yeh et al., 2006), *P. vannamei* (Cheng et al., 2008; Yeh et al., 2009), *M. japonicus* (Yeh et al., 2006; Cheng et al., 2008), *F. chinensis* (Liu et al., 2007a) and their role in shrimp humoral immune responses has been determined. *F. chinensis* transglutaminase (*Fc*TG) was reported to play role in immune response against bacteria. (Liu et al., 2007a).

2.3.3. Cytokines

Cytokines which play crucial role in innate and adaptive immunity had not been reported from crustaceans until Astakines (AST), a group of invertebrate cytokines, have been reported from shrimp, *P. monodon* and *P. vannamei*. Astakines play important role in hematopoiesis as well as have positive regulatory function against viral infection in shrimp (Hsiao and Song, 2010; Liang et al., 2015).

2.3.4. Antimicrobial peptides

AMPs, antimicrobial peptides or antimicrobial proteins, are widely considered to be the major antimicrobial effectors in humoral immunity effective against bacteria (Gram–positive as well as Gram–negative bacteria), yeasts as well as filamentous fungi, parasites and enveloped viruses (in few cases) (Imler and Bulet, 2005). In shrimp, different classes of AMPs or

effectors have been identified, and the most widely studied among them include Penaeidin (PEN), Crustin (Cru) and anti–LPS–factor (ALF). Other effectors functioning as AMPs identified in shrimp include C–type lectin (CTL), Lysozyme (Lyz), thioester–containing protein (TEP), stylicins and hemocyanin–derived proteins (Tassanakajon et al., 2018). Penaeidins, are a unique AMP family specifically identified in penaeid shrimp including *P. monodon, P. vannamei*, *F chinensis*, *M. japonicus* and *P. setiferus*, (Li et al., 2019; Tassanakajon et al., 2018). Penaeidins in general are active against bacteria (Gram–positive) and fungi (filamentous) (Tassanakajon et al., 2013). However, the possible role of penaeidins in antiviral immunity has also been reported based on studies of Pen5 from *P. monodon* (Woramongkolchai et al., 2011). Crustins and crustin–like sequences have been found in many shrimp species including *P. vannamei*, *P. monodon*, *F chinensis* and fresh water prawn *Machrobachium rosenbergii* (Li et al., 2019). A second group of secreted antimicrobial immune effectors reported within the Crustacea – the anti–lipopolysaccharide factors (ALFs) are small basic proteins which bind lipopolysaccharide and have strong antibacterial activity against, in particular, Gram–negative bacteria but also activity against Gram–positive bacteria (Amparyup et al., 2011). Other effectors functioning as AMPs identified in shrimp include C–type lectin (CTL), Lysozyme (Lyz), thioester–containing protein (TEP), stylicins and hemocyanin–derived proteins (Tassanakajon et al., 2018).

2.4. Pathogen recognition receptors (PRRs)

Innate immune responses are primarily mediated by germ–line encoded pathogen–recognition receptors (PRRs). These receptors recognise the molecular signatures associated with pathogens, which are invariant in nature, termed as pathogen–associated molecular patterns (PAMPs). Major PAMPs include lipopolysaccharides (LPS) and peptidoglycans (PGN) from Gram–negative and Gram–positive bacteria respectively, viral dsRNA (double–stranded RNA) and fungal β–glucans (GLU) (Imler and Zheng, 2004). After the recognition of these molecular patterns by PRRs, innate immune cells of the host initiate a wide–range of defence mechanisms that result in the development of inflammation and host resistance to infection (Akira et al., 2006). Pattern recognition receptor (PRR) families identified in shrimp, include lipopolysaccharide and β–1,3–glucan binding proteins (LGBPs), C–type lectins, galectins, thio–ester containing proteins (TEPs), Fibrinogen–related proteins (FREPs), scavenger receptors (SRs) , Down syndrome cell adhesion molecules (Dscams) and Toll–like receptors (TLRs). Some additional PRRs discovered

in shrimp, include the β–1,3–glucan binding proteins (BGBPs), serine protease homologue (SPHs) and trans–activation response RNA binding protein (TRBPs) (Wang and Wang., 2013). Among all the identified PRRs in shrimp, Toll–like receptors (TLRs) are recognised as major PRRs in shrimp innate immune system due to their critical role in host defence by recognition of pathogens (Sasai and Yamamoto, 2013; Li et al., 2019)

2.5. Signalling pathways regulating shrimp innate immunity

The innate immune response triggers a wide range of humoral as well as cellular activities via evolutionarily conserved signal transduction pathways that play a crucial role in antiviral as well as antibacterial defence both in *Drosophila* and humans (Borregaard, et al., 2000; Leulier and Lemaitre 2008). Since crustaceans including shrimps are closely related to insects, studying the expression profile of immune genes involved in immune responses in insects may be instrumental for understanding the immune responses of crustaceans, including penaeid shrimps especially *P. monodon* (Wilson et al., 2000). Studies on the expression profile of different genes in *D. melanogaster* after challenge with bacteria (Gram–positive and Gram–negative) as well as fungi have led to the identification of two major signalling pathways involved in innate immunity–The Toll pathway and the Immunodeficiency (IMD) pathway. Besides these pathways, another pathway, JAK/STAT pathway, has been identified in shrimp (Li and Xiang, 2013; Yan et al., 2015; Li et al., 2019). In insects, the Toll pathway is involved mainly in defence against Gram–positive bacteria, viruses and fungi and the IMD pathway functions in defence against Gram negative bacteria and virus, while the JAK/STAT pathway a role in defence against viruses. In shrimp the different components of these pathways form key regulators of the innate immune response. A vast majority of the genes or proteins involved in signalling pathways of *Drosophila* have also been identified and characterised in shrimp which suggests the existence of this pathway in shrimp and it has also been observed that these genes are responsive not only to bacterial infection by Gram–positive and Gram–negative bacteria, but also to viral infection with WSSV (Li, and Xiang, 2013; Li et al., 2019).

2.5.1. Toll–pathway

The Toll–pathway, which is the most widely studied among all signalling pathways, is known to play a key role in immune responses against pathogens by regulating

different genes coding for AMPs as well as other small peptides and components of the melanisation as well as clotting cascades (Li and Xiang, 2013). The TLR–MyD88–TRAF6–NF–κB signalling pathway is evolutionarily conserved in antibacterial and antiviral defense from *Drosophila* to humans (Kim and Ausubel, 2005; Lemaitre and Hoffman, 2007; Leulier and Lemaitre 2008). The components of the Toll–pathway in *Drosophila* include Spatzle, Toll, Pelle, Tube, Myeloid differentiation factor 88 (MYD88), Cactus, Dorsal, Dorsal–related immunity factor (DIF) (Belvin and Anderson 1996; Hoffmann and Reichhart, 2002; Hultmark, 2003; Lemaitre and Hoffmann 2007). In shrimp, most of the homologues of the components in the *Drosophila* Toll–pathway have been identified and characterised, including Toll–like receptors (TLRs), Spatzle, Tube, Pelle, TRAF6, Cactus, Dorsal, and their functions in shrimp innate immune responses against bacterial or viral infections has been deduced.

2.5.1.1. Toll–like receptors

The first Toll receptors (Toll–1) were discovered by its role in dorso–ventral patterning in *Drosophila* and were involved in the recognition of infectious non–self (Anderson et al., 1985). Subsequently, many researchers demonstrated the existence of Toll/TLR proteins in other invertebrates and in vertebrates (Medzhitov et al., 1997; Tauszig et al., 2000; Beutler, 2004; West et al., 2006). Moreover, its importance as a PRR was further confirmed by studies on innate immune system in mammals, which revealed the existence of TLRs in mammals and their role in direct recognition of infectious non–self (Underhill et al., 2002). According to Kawai and Akira, (2010) TLRs are glycoproteins (type–I protein) containing a transmembrane domain, an extracellular N–terminus with leucine–rich repeats (LRRs) responsible for the recognition of PAMPs, and an intracellular C–terminus with a TIR domain (Toll/interleukin–1 receptor domain) required for downstream signal transduction. Various studies have also reported the association of TLRs in the synthesis of anti–microbial peptides, and the essential role it plays in immunity against fungal and Gram–positive bacterial infection as well as antiviral response in *Drosophila* (Lemaitre et al., 1996; Rutschmann et al., 2002; Zambon et al., 2005). Among the different PRRs identified so far, Toll and/or Toll–like receptors (TLRs) are considered as canonical molecules involved in pathogen–recognition in metazoans (Tauszig et al., 2000; Uematsu and Akira, 2006) and the TLR family is reported to be widely conserved during evolution (Kawai and Akira, 2010). Among shrimps, the first partial cDNA sequence of Toll–like receptor was reported

by Arts et al. (2007) in in tiger shrimp, *P. monodon* and was designated as *Pm*Toll. Subsequently, full–length cDNAs of different isoforms of Toll–like receptors, including complete sequence from *P. monodon* (*Pm*Toll) and *Pm*Toll–9 (Assavalapsakul and Panyim, 2012; Liu et al., 2018), nine types of Toll genes from *P. vannamei* (*Lv*Toll 1–9) (Yang et al., 2007; Wang et al., 2012; Li et al., 2018), *Fc*Toll from *F. chinensis* (Yang et al., 2008), *Mj*Toll1 and *Mj*Toll2 from *M. japonicus* (Mekata et al., 2008) and Tolls from freshwater prawns *M. rosenbergii* (Srisuk et al.,2014; Feng et al.,2016) as well as from *M. nipponense* (Pan et al., 2019) have been identified. Wang and Wang (2013) had categorised the shrimp TLRs (Tolls) into three groups on the basis of sequence identities and phylogenetic analysis: *Pm*Toll1, *Fc*Toll1, *Lv*Toll1 and *Mj*Toll1 and belong to type 1 TLR; *Lv*Toll2 and *Mj*Toll2 belong to type 2 TLR; and *Lv*Toll3 belongs to type 3 TLR of shrimps. The identified shrimp TLRs contain typical structural or functional motifs of the TLR family, Death domain at the N–terminal and a cytoplasmic TIR signalling domain at the C–terminal and they share homology with *Drosophila* Tolls (Li and Xiang, 2013).

2.5.1.2. Spatzle

Spatzle is a cytokine–like molecule which acts as an extracellular ligand for Toll to facilitate recognition of PAMPs from pathogens. In order to bind to Toll and activate the toll pathway, Spatzle is enzymatically cleaved from an inactive pro–Spatzle which contains a prodomain preventing the binding of Toll receptor and Spatzle. In *Drosophila*, the interaction between the activated Spatzle–containing C terminal domain (C–106) and the Toll–extracellular domain rearranges the conformation of the cytoplasmic TIR domain, to generate a docking site for recruiting adaptors such as MyD88 through their TIR–domain, which subsequently result in the signalling cascade (DeLotto and DeLotto, 1998). In shrimp, the first Spatzle–like protein was identified in *F. chinensis* (*Fc*Spz) and it was detected that the activated form of *Fc*Spz is composed of seven cysteine residues on C–terminal 114 amino acids, and these cysteine residues are essential for the intra– and inter– molecular disulphide bonds for formation of Spatzle homodimers (Shi et al., 2009). In their study, Shi et al., (2009) reported that *Fc*Spz responds to *Vibro alginolyticus* as well as WSSV infection and its active form, the recombinant *Fc*Spatzle C–114, can trigger Crustin 2 AMP expression of in crayfish. Subsequently, Spatzle has been identified from shrimps and their response to viral/bacterial infection has been determined (Li et al., 2019). These include *Lv*Spz1–4 from *P. vannamei* (Wang et al., 2012; Yuan et al., 2017),

*Pm*Spz 1 from *P. monodon* (Boonrawd et al., 2017), *Mj*Spz from *M. japonicus* (Ren et al., 2017) and *Mr*Spz from *M. rosenbergii* (Vaniksampanna et al., 2019).

2.5.1.3. Canonical components of the Toll–pathway

Once TLR recognises PAMPs, a signalling cascade is triggered employing the components of the Toll–pathway and this signalling cascade has been well studied in *Drosophila*. Spatzle binds to Toll and the activation of Toll causes dimerisation of intracytoplasmic TIR domains further recruiting the key adaptor molecule MyD88 through its own TIR domain. (Tauszig–Delamasure et al., 2002). Tube (Interleukin–1 receptor–associated kinase–4 (IRAK–4) homologue), another adaptor molecule binds with MyD88, and Pelle (Interleukin–1 receptor–associated kinase–4 (IRAK–1) homologue) through pairwise interactions of their respective death domains (Sun et al., 2004; Ji et al., 2014). Pelle, upon autophosphorylation interacts with downstream molecules resulting in the activation of tumour necrosis factor receptor–associated factor 6 (TRAF6) and destruction of inhibitor of Kappa B (IκB), Cactus (Valanne et al., 2011). Cactus (IκB) functions as an inhibitor of Dorsal (Nuclear factor KappaB – NF–κB) and contains an N–terminal serine rich regulatory region involved in ubiquitin recognition and proteasomal degradation, along with ankyrin repeats which can bind to the Rel–homology (RHD) region of Dorsal and a PEST (proline–glutamic acid– serine –threonine–rich) domain that acts as a destabilising domain for inhibiting the binding of DNA (Ernst et al., 1995; Chen and Greene, 2004). Destruction of Cactus further leads to translocation of Dorsal or Dorsal–related immunity factor (DIF) (Lindsay and Wasserman, 2014).

Many studies have suggested the existence of TLR/MyD88/Tube/Pelle/TRAF6/ NF–κB cascade in shrimps for regulation of immune gene which have been reviewed by Li and Xiang (2013) and more recently by Li et al. (2019) and the Toll –pathway signalling cascade is similar to that identified in *Drosophila*. In shrimp full–length cDNAs of the Toll–pathway genes have been identified and characterised in response to pathogens. MyD88 homologues with the characteristic TIR domain and Death domain have been identified from *P. vannamei* (*Lv*MyD88 and *Lv*MyD88–1) (Zhang et al., 2012) and from *F. chinensis* (*Fc*MyD88) (Wen et al., 2013). Tube (IRAK–4) and Pelle (IRAK–1) genes have been identified from *P. vannamei* (*Lv*Tube, *Lv*Tube–1, *Lv*Pelle) (Li et al., 2014) and Tube from *P. monodon* (*Pm*IRAK–4) (Watthanasurorot et al., 2012)

and pelle (*Fp*IRAK1) from *Fenneropenaeus penicillatus* (Xu et al., 2017) ; TRAF6 from *P. vannamei* (*Lv*TRAF6) (Wang et al. 2011) and *F. penicillatus* (*Fp*TRAF6) (Cai et al., 2017). Cactus (IκB) has been identified in *P. vannamei* (*Lv*Cactus) and in *F. chinensis* (*Fc*Cactus) (Li et al., 2012; Wang et al., 2013a) and from freshwater prawn *M. rosenbergii* (*Mr*NFƙBI–α) (Arockiaraj et al., 2012). Dorsal homologues reported in shrimp include, *Fc*Dorsal, *Lv*Dorsal, were characterised from *P. vannamei* and *F. chinensis* as well as *Mr*Dorsal and *Mn*Dorsal were characterised from freshwater prawns *M. rosenbergii* and *M. nipponense* (Huang et al., 2010; Li et al., 2010; Huang et al., 2016a; Huang et al., 2020). Apart from these classical components of Toll pathway, some components involved in regulating the Toll– pathway directly or indirectly have been identified from *P. vannamei* which include Tollip (*Lv*Tollip), sterile–alpha and armadillo motif–containing protein (*Lv*SARM), Flightless–I (LvFli–I) (Wang et al., 2013b; Wang et al., 2013c; Zhang et al., 2015) and ß–arrestins from *M. japonicus* (*Mj*barr1–2) (Sun et al., 2016).

2.5.2. IMD pathway

The IMD pathway was first described in *Drosophila* based on a mutation named as immune deficiency (IMD) which impaired production of AMPs in mutant flies upon bacterial infection mainly with Gram negative bacteria (Lemaitre et al., 1995). The canonical components identified in this pathway in *Drosophila* include– IMD, the peptidoglycan recognition protein–LC (PGRP–LC) and PGRP–LE receptors, the TGF–β –activated kinase 1 (TAK1), TAK1–associated binding protein (TAB2), the dFADD adaptor (*Drosophila* Fas–Associated protein with death domain), the caspase–8 homologue Death–related ced–3/Nedd2–like protein or DREDD caspase, the inhibitor of κB kinase (IKK) complex or the IKK signalosome consisting of Ird5 (mammalian IKKβ homologue) and Kenny (IKKγ homologue), the IAP2 (the ubiquitination machinery components inhibitor of apoptosis 2) the NF–κB transcription factor–Relish as well as the transcription cofactor Akirin and the ubiquitin conjugating enzymes Bendless (Ubc13), Uev1a as well as Effete (Ubc5) (Kleino and Silverman, 2014). The IMD pathway in *Drosophila* recognises Gram–negative bacteria and is also involved in antiviral immune responses (Lemaitre et al., 1995 Costa et al., 2009). In shrimps the first identified components include IMD and Relish (Li and Xiang, 2013; Li et al., 2019). The first complete cDNA sequence of an IMD homologue in shrimp was identified from *P. vannamei*. (*Lv*IMD) by Wang et al. (2009). Further IMD

homologue was identified and characterised from *F. chinensis* (Lan et al., 2013; Feng et al., 2014) and more recently from freshwater prawn *M. nipponense* (Li et al., 2018). Relish, which is a Rel/NF–kB transcription factor, is activated by the IMD pathway in response to Gram–negative bacterial infection and regulates the expression of majority of the *Drosophila* AMPs. Li et al. (2009) identified a Relish homologue (designated *Fc*Relish) and obtained its full–length cDNA sequence. Simultaneously, two Relish homologues *Lv*Relish and *sLv*Relish (a short spliced isoform) were identified from *P. vannamei*, *P. monodon* and freshwater prawn *M. rosenbergii* (Huang et al. 2009; Visetnan et al., 2014; Shi et al., 2015). Following the identification of IMD and Relish, many more components of IMD pathway have been identified in shrimp which include TAK1, TAB1, TAB2, Relish, IKK homologues (IKKβ and IKKε), mitogen–activated protein kinases (MKK3, MKK4, MKK6, MKK7), c–Jun N–terminal kinase (JNK), c–Jun, c–Fos, p38, ATF2, NF–κB repressing factor (NKRF), Akirin, Bap60, and 14–3–3 (Li et al., 2019).

2.5.3. JAK/STAT pathway

Apart from the Toll and IMD signalling pathways, the JAK/STAT signalling pathway has also been identified as a significant pathway in innate immunity of invertebrates, however, there are relatively fewer studies on this pathway. The JAK/STAT pathway has been reported to be involved in antiviral immune response in *Drosophila* and the components of this pathway include – three extracellular unpaired ligands (UPD1, UPD2, UPD3), a cytokine–like transmembrane receptor Domeless (DOME), Janus tyrosine kinase (JAK) Hopscotch or Hop and the transcription factor STAT (Signal transducer and activators of transcription) named STAT92E (Agaisse and Perrimon 2004; Dostert et al., 2005; Myllymaki and Ramet, 2014). The three unpaired ligands bind to the Dome receptor to mediate signal transduction, following this JAKs and their associated receptors are phosphorylated leading to the recruitment of STAT via their characteristic SH2 (Src–homology 2) domain and subsequent phosphorylation of STAT (Arbouzova and Zeidler 2006; Li 2008). Further the phosphorylated STATs form homo or hetero–dimers and translocate to the nucleus from the cytoplasm and act as transcriptional activators for specific genes for mediating various responses involved in cell growth as well as differentiation and also different immune responses (Li 2008, Murray, 2007). Different components of the JAK/STAT pathway have been identified in shrimp and their antiviral responses have been studied indicating the existence of this pathway in shrimp (Chen et al., 2008; Okugawa et al.,

2013; Yan et al., 2015; Song et al., 2015). The first STAT in shrimp was reported from *P. monodon*, which was also the first STAT reported among crustaceans and it was reported that WSSV activates STAT which benefits replication of virus (Liu et al., 2007b; Chen et al., 2008). Following the identification of *Pm*STAT, STAT homologues have been characterised from *P. vannamei* (Wen et al., 2014; Yao et al., 2016), *F. chinensis* (Sun et al. 2011) *M. japonicus* (Okugawa et al., 2013) and freshwater prawn *M. rosenbergii* (Huang et al., 2016b). Moreover, JAK and Dome have been identified and characterised from *P. vannamei* (Song et al., 2015; Yan et al., 2015) and full length cDNA sequences of these genes have been identified from *M. japonicus*. Different homologues of the negative regulators of the pathway previously identified in *Drosophila* namely SOCS and PIAS have been identified from shrimp *M. japonicus Mj*SOCS (Okugawa et al., 2013) *Mj*SOCS2 (Sun et al., 2016) and *Mj*PIAS (Niu et al., 2018) as well as *P. vannamei Lv*SOCS2 (Wang et al., 2016) and *Lv*PIAS (Zhang et al., 2019).

2.6. Major diseases affecting shrimps

2.6.1. White spot syndrome virus

Shrimp culture has been adversely affected by diseases caused by viruses including white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHNNV), Taura syndrome virus (TSV) and yellow head virus (YHV) (Walker and Winton, 2010). However, the greatest impact on shrimp farming has been due to WSSV that emerged as a major disease problem and still continues to be a serious threat to the shrimp culture industry (Flegel, 2006, Stentiford et al., 2012; Tandel et al., 2017). WSSV belongs to the Genus, *Whispovirus*; Family, Nimaviridae, and is a rapidly replicating large, enveloped, dsDNA virus with high virulence causing white spot disease in shrimp and leading to huge mortalities . Almost every species of penaeid shrimp is susceptible to WSSV (Lightner, 1996). WSSV, the most pathogenic among viruses infecting penaeid shrimp, has had huge impact on the sustainability as well as progress of the worldwide shrimp farming industry, owing to its high virulence and its ability to infect, cause disease and mortality in a wide–range of crustaceans, including crayfish (Jiravanichpaisal et al., 2001, Edgerton, 2004), crabs (Rajendran et al., 1999), lobsters (Chang et al., 1998; Rajendran et al., 1999), and *Macrobrachium* species (Rajendran et al., 1999; Kiran et al., 2002). White spot disease is characterised by the presence of

white spots on the inner surface of the exoskeleton and other clinical signs include anorexia, lethargy and reddish discoloration of the body (Walker and Winton, 2010). The main targets for replication of WSSV are tissues of ectodermal as well as mesodermal origin and include gills, cuticular epithelium lymphoid organ, hematopoietic tissues, connective tissue of hepatopancreas , ovary, antennal gland , stomach the ventral nerve cord (Wang et al., 1995; Wongteerasupaya et al., 1995; Lo et al., 1997). Several methods including microscopy, bioassay, electron microscopy, histopathological, immunological and molecular methods have been developed in order to detect infection caused by WSSV (Lightner and Redman, 1998). Histopathological changes in the target tissues such as Cowdry A type inclusions in the early stage and basophilic inclusion bodies and other cellular changes associated with the late stage of infection were used as diagnostic features of WSSV (Lightner, 1996). Among the molecular diagnostic methods, polymerase chain reaction (PCR) has been the most widely used technique and includes single step PCR (Lightner 1996; Takahashi et al., 1996), two–step PCR (Tapay et al., 1999; Hossain et al., 2004), semi–nested PCR (Kiatpathomchai et al., 2001), quantitative competitive PCR (Tang and Lightner, 2000) and quantitative real–time PCR (Dhar et al., 2001). Crustacean cell culture system as possible *in vitro* models for virus replication, have gained momentum especially due to viral diseases affecting commercially important crustaceans and there have been many experimental studies on replication of WSSV in crustacean primary cultures– including haemocyte and lymphoid organ cultures from *P. monodon* (Jose et al., 2010; Jose et al., 2012); haemocyte cultures from *F. chinensis* (Jiang et al., 2006), lymphoid organ and ovarian cultures from *M. japonicus* (Itami et al., 1999; Maeda et al., 2004); haemocyte cultures of mud crab *Scylla serrata* (Deepika et al., 2014)

2.6.2. Vibriosis

Vibriosis, caused by Gram–negative bacteria in the family Vibrionaceae, is responsible for mortality of farmed shrimp worldwide and is one of the major disease problems in shellfish and finfish aquaculture (Lavilla–Pitogo et al., 1998, Thitamadee et al., 2016). Many *Vibrio* sp. comprise the naturally present microflora in shrimp in the wild as well as in culture systems however, being opportunistic pathogens they become pathogenic during suppressed host defense system under conditions of stress. Several *Vibrio* species have been associated with disease outbreaks in shrimp including the main pathogens *Vibrio harveyi as well as V.*

parahaemolyticus and other species – V. alginolyticus, V. anguillarum, V. campbellii, V. damselae (Photobacterium damselae), V. cholera (non –01 serotype), *V. vulnificus, V. penaeicida, V. nereis, V. owensii, V. splendidus,* etc. Among the different *Vibrio* species identified as shrimp pathogens, one of the most important is the Gram–negative *Vibrio harveyi,* a luminous bacterium causing luminescent vibriosis in penaied shrimps (Lavilla–Pitogo et al., 1990), which is associated with mass mortalities in *P. monodon* larvae (Karunasagar et al., 1994). The mortality rate has been recorded as high as 80–100% in hatcheries of *P. monodon* since *V. harveyi* can invade the eggs as well as larvae (Karunasagar et al., 1994) with mortalities also reported in juvenile shrimp. *V. harveyi* colonises the digestive tract and is adapted to survive in the acidic conditions of shrimp gut (Soonthornchai et al., 2015). Hepatopancreas is the main target organ of *V. harveyi* and upon infection the bacteria is known to cause severe inflammation of hepatopancreas tubules, disruption of the tissues of the hepatopancreas as well as gut of shrimp (Leano et al 1998; Jiravanichpaisal et al., 2010). Apart from the most common disease of luminescent vibriosis, *V. harveyi* is associated with several disease conditions in shrimp including diseases of eye (Smith, 2000), detachment of epithelial lining of mid gut and pathological changes in gut (Martin et al., 2004), blockage of digestive tracts by balled–up tissues of the epidermis (bolitas negricans) (Robertson et al. 1998; Austin and Zhang, 2006), red leg or red disease (Alapide–Tendencia and Dureza, 1997), black shell disease (Joseph et al., 2005), Loose shell syndrome, white gut disease and red disease (Jayasree et al., 2006), bright–red syndrome characterised by appearance of red spots in abdomen (Soto–Rodriguez et al., 2010), white–tail disease characterised by white lesions in the tail due to muscle necrosis (Zhou et al., 2012) and recently it has been also reported to be associated with the devastating acute hepatopancreatic necrosis disease (AHPND) (Muthukrishnan et al., 2019). In the last few years, there have been several reports on the increasing economic impact of early mortality syndrome (EMS), which has been named as acute hepatopancreatic necrosis disease (AHPND) (Tran et al., 2013, Thitamadee et al., 2016; Santos et al., 2020). A unique isolate of *Vibrio parahaemolyticus* (VP_{AHPND}) has been reported to be the causative agent of AHPND and this isolate contains a 69 kb plasmid pVA–1 carrying binary Photorhabdus insect–related (Pir) –like toxin genes pirA and pirB (Han et al., 2015). VP_{AHPND} colonises the shrimp stomach and release toxins into the hepatopancreas which result in the sloughing–off, necrosis of the tubule epithelial cells (Tran et al., 2013). Infection with AHPND can lead to sudden mass mortalities in shrimps up to 100% within 30–35 days of stocking and *P.*

monodon as well as *P. vannamei* are the most susceptible species (Tran et al., 2013; de la Pena et al., 2015; Santos et al., 2020). Vibriosis is a common problem world–wide, particularly in India, with *V. harveyi* and *V. parahaemolyticus* being the main pathogens infecting *P. monodon*. Study of immune gene expression in different tissues in response to *Vibrio* infections is vital in understanding immune mechanisms in shrimp and for developing shrimp health monitoring protocols

2.7. Expression of innate immune genes in shrimp

Different immune genes of the signalling pathways have been characterised in shrimp and their expression in healthy animals and upon pathogen challenge has been elucidated. Expression of immune–related genes in different developmental stages have been reported from diverse groups of organisms (Tirape et al., 2007; Zhang et al., 2010; Puill–Stephan et al., 2012; Balseiro et al., 2013; Caipang and Fagutao 2013; Cecchini et al., 2013; Hines et al., 2014). Ther are few studies on expression of different immune–relevant genes in the larval stages of shrimps is limited (Jiravanichpaisal et al., 2007, Yang et al., 2010; Yao et al., 2010; Martin et al., 2012; Rajkumar et al., 2017). Moreover, Quispe et al. (2016) studied different immune relevant genes during ontogenesis of *P. vannamei,* including Dorsal of Toll pathway and Relish of IMD pathway. In another study by Rajkumar et al., (2017), the expression of *Lv*TRAF6 was determined in nauplii, protozoeae, mysis, early post–larvae (PL5–10) and late post–larvae (PL11–20) using RT PCR and real–time PCR. *Lv*TRAF6 was detected in all stages except for naupliar stage wherein no expression of the gene was detected using RT PCR and slight expression was detected by real–time PCR. The expression of STAT, a component of the JAK/STAT pathway was detected in all the developmental stages, including nauplii, mysis, and post larvae of *F. chinensis* (*Fc*STAT) (Sun et al., 2011). Although there are few studies on ontogenesis of genes in the signaling pathways, the tissue–level expression of these genes in healthy shrimp has been studied by many researchers. Moreover, expression profiles of genes in signalling pathways of shrimp have been determined after pathogen challenge or ligand stimulation by some researchers. The expression of Spatzle genes *Fc*Spz from *F.chinensis* (Shi et al., 2009), *Pm*Spz1 from *P. monodon* (Boonrawd et al., 2017) and *Lv*Spz4 from *P. vannamei* (Yuan et al., 2017) were reported in various tissues including haemocyte, heart, hepatopancreas, gill, stomach as well as intestine and their expression kinetics in response to WSSV and bacteria have been elucidated. *Pm*Toll expression in different

tissues of healthy shrimp and upon exposure to WSSV, have been reported by Arts et al. (2007) and Assavalapsakul and Panyim (2012). Expression analysis of *Fc*Toll in lymphoid organ of shrimp after *Vibrio* or WSSV stimulation was carried out by Yang, et al. (2008). Mekata, et al. (2008), reported the *Mj*Toll gene expression in lymphoid organ stimulated with peptidoglycan. The transcription modulation of *Lv*Toll of *L. vannamei* after *Vibrio* and WSSV challenge has been reported by Wang et al. (2012). Constitutive expression of the shrimp MyD88, TRAF6, Tube (IRAK–4), Pelle (IRAK–1) and Cactus have been reported in different tested tissues. In a study on MyD88 expression in *F. chinensis* in response to WSSV, it has been reported that the transcription level of *Fc*MyD88 mRNA remained unchanged while the protein level of *Fc*MyD88 was up–regulated at few time–points (Wen et al., 2013). Moreover, up–regulated expression of *Lv*MyD88 in haemocytes of *P. vannamei* upon challenge with WSSV and bacteria has been reported by Zhang et al. (2012). The role of IRAK4 in the intestinal immunity of *P. monodon* after bacterial challenge as well as its normal expression in different tissues has been reported (Watthanasurorot et al., 2012). After studying the expression of *Lv*TRAF6 in *V. alginolyticus* and WSSV–challenged shrimp, Wang et al. (2011) indicated the possible role of *Lv*TRAF6 in antibacterial and antiviral responses. The expression levels of the downstream molecule cactus in *P. vannamei* (*Lv*Cactus) increased in response to bacteria and decreased in response to WSSV as reported by Li et al., (2012). *Fc*Cactus expression was also modulated after bacterial challenge (Wang et al., 2013a). Li et al. (2010) reported the transcriptional expression of *Fc*Dorsal in the haemocytes and lymphoid organ in shrimp stimulated with bacteria or WSSV. Huang et al. (2010) reported that *Lv*Dorsal mRNA was expressed in all the tissues tested including gill, epidermis, haemocytes, intestine, stomach, eyestalk, brain, hepatopancreas, muscle, heart and pyloric caecum. They also recorded the expressions of *Lv*IMD mRNA in different tissues after LPS (or Gram–negative bacteria) and WSSV exposure and indicated the possible role of *Lv*IMD in shrimp immune response to Gram–negative bacteria and virus. Components of the JAK/STAT pathway have been reported to play important role in immune response against virus infection in shrimp and other crustaceans. Chen et al. (2008) reported that *Pm*STAT was exploited by WSSV to benefit its replication and in the same study they reported a significant decrease in shrimp STAT expression in cephalothorax of *P. vannamei* upon WSSV challenge. The up–regulated expressions of *Fc*STAT in *F. chinensis* (Sun et al., 2011) as well as *Mr*STAT in *M. rosenbergii* (Huang et al., 2016b) and *Mj*STAT in *M. japonicus* (Ren et al., 2015) indicate the positive role of shrimp

STAT in antiviral immunity. In *P. vannamei*, constitutive expression of JAK (*Lv*JAK), Domeless (*Lv*DOME), SOCS2 (*Lv*SOCS2) and PIAS (*Lv*PIAS) was observed in haemocyte, gill, heart, stomach, hepatopancreas, intestine, epithelium, eyestalk, muscle, pyloric caecum and scape.. *Lv*JAK and *Lv*DOME also showed up–regulation expression in response to WSSV but their expression was mostly unaffected in response to *V. parahaemolyticus* (Song et al., 2015; Yan et al., 2015). However, the negative regulators of the JAK/STAT pathway in shrimp *Mj*SOCS2, *Mj*PIAS, *Lv*SOCS2 and *Lv*PIAS could be significantly induced by bacterial infection (Wang et al., 2016; Sun et al., 2016b; Niu et al., 2018; Zhang et al., 2019). Apart from invivo studies, ligand–specificity studies using poly I:C, LPS, peptidoglycan of some these genes have also been carried out in primary cell cultures of mud crab (Vidya et al., 2014) and scallop (Qiu et al., 2007; Qiu et al., 2009). These evidences suggest that a relationship might exist among Toll pathway, IMD pathway, JAK/STAT pathway and WSSV/bacterial infection, however further studies have to be carried out to support this. Studies have also been carried out to determine expression of other immune–related genes upon pathogen challenge. Soonthornchai et al. (2010) used juvenile *P. monodon* as a model to study the effect of infections caused by *V. harveyi* on immune gene expression in the intestine through natural infection routes. With the application of high throughput screening approach like SSH library construction, immune related genes in response to *V. harveyi* infection have been analysed in challenged *P. monodon* (Nayak et al., 2010).

3. MATERIAL AND METHODS

3.1. Cloning and sequencing of potential new immune genes in *Penaeus monodon*

The selected immune genes were subjected to PCR amplification using specific primers which were designed from the conserved regions of those reported from other crustaceans. The amplified cDNA fragments were purified by gel extraction (GeneJET gel extraction kit, Thermo Scientific, USA), ligated with pTZ57R/T vector and subsequently cloned in DH5α strain of *Escherichia coli* by TA cloning using InsTAclone™ PCR cloning Kit (Thermo Scientific, USA). Plasmid DNA was isolated from the positive clones using GeneJET plasmid miniprep kit, (Thermo Scientific, USA) and sequenced using sequencing services of Eurofins Genomics Pvt. Ltd. (Bangalore, India). The full–length sequence of *Pm*Spatzle, *Pm*MyD88 and *Pm*TRAF6 were obtained by 5'– and 3'– RACE, using the SMARTer^{TM} RACE cDNA

Amplification kit (Clontech, USA) according to the manufacturer's instructions. Partial sequences of *Pm*Cactus and *Pm*Dorsal were also obtained by PCR amplification and by 5'–RACE.

3.2. Normal expression of selected immune genes in *Penaeus monodon*

Available crustacean immune gene sequences in the GenBank were analysed and specific primers were designed for the selected immune genes namely *Pm*Toll, *Pm*Spatzle, *Pm*MyD88, *Pm*IRAK–4, *Pm*TRAF6, *Pm*Cactus and *Pm*Dorsal. Different developmental stages of shrimp, *P. monodon* (egg, nauplii, zoea, mysis and post–larvae) were collected from Vasavi Hatchery, Kakinada (India). Healthy adult *Penaeus monodon* were collected from a local farm and maintained in the laboratory in optimum conditions. Different tissues including haemocytes, gill, muscle, lymphoid organ, heart, hepatopancreas, stomach, midgut, hindgut, thoracic ganglion, abdominal ganglion, cephalic ganglion, pleopod, epidermal membrane and eye stalk were collected for studying tissue level expression. The samples were processed for RNA isolation followed by cDNA synthesis and subjected to real time PCR using specific primers.

3.3. Challenge with WSSV and *Vibrio harveyi in vivo*

In the challenge experiments, healthy *P. monodon* were injected intramuscularly in the third abdominal segment with 100 µL of WSSV inoculum (1.3×10^3 copies/µL) or 100 µL PBS suspension of *Vibrio harveyi* (1×10^6 CFU/mL) . Un–injected shrimp were used as control. Three animals from each group were selected for sampling, and seven tissues including gill, lymphoid organ, stomach, hepatopancreas, midgut, hindgut and haemocytes were collected from each challenged animal. Samples were collected at 2, 6, 12, 24, 48 and 72 h post–injection. The tissues were further processed for RNA isolation and cDNA synthesis and subjected to real time PCR based relative expression analysis using Elongation factor–1α (EF–1α) as internal reference gene.

3.4. Immune gene expression *in vitro* in response to selected ligands and WSSV

Primary cell cultures were developed from haemocytes of *P monodon.* Aliquots of the haemocyte suspensions seeded in 6–well plates (2×10^6 cells/well) were exposed to 200 µL WSSV inoculum (2.4×10^2 copies/µL). 50 ng poly I: C, 10 µg each of peptidoglycan and

lipopolysaccharide, separately. Un–exposed cells (cells treated with serum–free medium) were used as control. Haemocytes were harvested at 2, 6, 12, 24, 48 and 72 h time–points, processed for RNA isolation then cDNA synthesis and subjected to real time PCR based relative expression analysis using EF–1α as internal reference gene.

4. RESULTS

4.1. Identification and characterisation of possible new immune gene/s in *Penaeus monodon*

Immune genes namely *Pm*Spatzle, *Pm*MyD88, *Pm*TRAF6, *Pm*Cactus, *Pm*Dorsal, which were previously not characterised in *P. monodon* were identified and their partial/complete cDNA sequence was determined. Complete cDNA sequence of *Pm*Spatzle is 1195 nucleotide (nt) with an open reading frame (ORF) of 741 nt encoding a putative protein of 246 amino acids (aa) consisting of a 5' –untranslated region (UTR) of 214 nt, and 3' UTR of 241 nt with a poly (A) tail. The highly conserved C–terminal activated 'Spaetzle' domain was identified in the putative *Pm*Spatzle protein at the position 140–239 aa by SMART. *Pm*Spatzle full–length deduced amino acid sequence showed highest sequence identity with shrimp spatzles: *P. vannamei* Spz3 (93%) followed by *F. chinensis* (92%) and showed 77% sequence identity with *M. rosenbergii* Spatzle and was closely clustered with the shrimp spatzles in the phylogenetic tree. The full–length sequence of *Pm*MyD88 is 1716 basepairs (bp) long with an ORF of 1449 bp encoding a putative protein of 482 amino acids (aa), a 5' untranslated region (UTR) of 136 bp, and a 3' UTR of 131 bp with a poly (A) tail. Sequence analysis predicted a Death domain (residues 31–104) and a TIR domain (residues 157–349) and C–terminal extension (CTE) domain following the TIR domain in the deduced amino acid sequence. Homology analysis by BLASTP search indicated that *Pm*MyD88 full–length deduced amino acid sequence exhibited 98% similarity with MyD88 from *F. chinensis*, 97% and 86% with different variants of MyD88 from *P. vannamei*, while 67–70% sequence identities were detected with MyD88s from crabs, *Eriocheir sinensis*, *Scylla serrata*, *S. paramamosain* and *P. trituberculatus.*In the phylogenetic tree *Pm*MyD88 was closely clustered with *Lv*MyD88 and *Fc*MyD88. The full–length cDNA of *Pm*TRAF6 is 2563 bp long with an ORF of 1785 bp encoding a putative protein of 594 amino acids Sequence analysis indicated that *Pm*TRAF6 contains an N–terminal RING–type zinc finger domain of 39 aa (residues 31–69), two

TRAF–type zinc finger domains of 56– and 58– aa (residues 113–168 and 168–225, respectively), and a conserved C–terminal meprin and TRAF homology (MATH) domain of 125 aa (residues 419–543). The full–length *Pm*TRAF6 putative protein shared the highest identity of 98% with *F. chinensis* and *F. penicillatus* followed by 97% identity with *P. vannamei* and *M. japonicus* TRAF6, as revealed by BLASTP analysis and was closely clustered with shrimp TRAF6 as revealed by phylogenetic analysis. The identified *Pm*Cactus cDNA partial sequence consisted of 1534 bp with an ORF coding for 463 aa. Analysis of the protein domains by SMART revealed six ankyrin repeats (ANKs) distributed in the 175–398 region. *Pm*Cactus showed 97% similarity with *Fc*Cactus from *F. chinensis* and 99% with *Lv*Cactus from *L. vannamei*. *Pm*Dorsal cDNA partial sequence consisted of 1155 nt with an ORF coding for 339aa. Analysis of the protein domains by SMART revealed a Rel homology domain (RHD) in the 82–252 region which is a common feature of the NF–κB family proteins and is located at the N–terminus of the protein. *Pm*Dorsal showed 99% similarity with both *Fc*Dorsal and *Lv*Dorsal. Both *Pm*Cactus and *Pm*Dorsal were closely clustered with their respective homologues in shrimp in the phylogenetic tree.

4.2. Normal expression of identified immune genes

4.2.1. Expression of immune genes in different developmental stages

The expression of the selected immune genes was determined in early developmental stages of *P. monodon*. The selected immune genes such as *Pm*Toll, *Pm*Spatzle, *Pm*MyD88, *Pm*IRAK–4, *Pm*TRAF6, *Pm*Cactus and *Pm*Dorsal were ubiquitously expressed with varying levels in egg, nauplii (1,2,3), zoea (1,2,3), mysis (1,2,3) and post–larvae (PL1-,5,7,10,14,18) of *P. monodon* as observed in real–time PCR using EF–1α as internal reference gene. *Pm*Toll expression showed the highest level in PL4, followed by PL10, PL7 and eggs whereas the maximum expression of *Pm*Spatzle was noted in nauplii–2 stage. *Pm*MyD88 and *Pm*IRAK–4 showed maximum expression in eggs. Similarly, expression level of *Pm*TRAF6 was highest in eggs. The gene expression of *Pm*Cactus was found to be highest in zoea2 and minimum in PL2 whereas expression of *Pm*Dorsal was found to be the highest in zoea3 and minimum in PL5.

4.2.2. Tissue–level expression

The expression of the immune genes namely *Pm*Toll, *Pm*Spatzle, *Pm*MyD88, *Pm*IRAK–4, *Pm*TRAF6, *Pm*Cactus, *Pm*Dorsal, was determined in 15 tissues including gill,

lymphoid organ, heart, stomach, hepatopancreas, midgut, hindgut, epidermis, pleopods, eyestalk, cephalic ganglion, abdominal ganglion, thoracic ganglion, muscle and haemocytes. The selected immune genes were found to be ubiquitously expressed with varying levels in all the 15 selected tissues of *P. monodon* as observed in quantitative real–time PCR using EF–1α as internal reference gene. *Pm*Toll showed the highest expression in lymphoid organ and the lowest expression in muscle. In the case of *Pm*MyD88, the lowest expression was noticed in muscle, while midgut tissue showed the highest expression. Eyestalk tissue exhibited the highest expression of *Pm*TRAF6 and *Pm*Spatzle while the minimum expression was observed in haemocytes and thoracic ganglion respectively. The maximum expression of *Pm*IRAK–4 was observed in haemocytes. In the case of *Pm*Cactus and *Pm*Dorsal the highest and lowest expressions were found in midgut and lymphoid organ and cephalic ganglion and hepatopancreas, respectively.

4.3. In–vivo expression profiles of the selected immune genes in response to WSSV and *Vibrio harveyi* infection

Expression level of selected immune genes in response to pathogen (WSSV and *Vibrio harveyi*) challenge was studied by real time PCR in seven selected tissues namely gill, lymphoid organ, stomach, hepatopancreas, midgut, hindgut and haemocytes. The expression was analysed in the selected tissues collected at different time–points post–challenge *viz*., 2, 6, 12, 24, 48 and 72h. The genes showed modulation in expression upon infection.

In response to WSSV, *Pm*Toll showed significant up–regulation at all time–points of infection in most of the tissues. *Pm*Spatzle was significantly up–regulated in haemocytes and showed elevated expression in hepatopancreas at some early time–points. WSSV caused significant up–regulation of *Pm*MyD88 in most of the tissues tested. *Pm*IRAK–4 was significantly up–regulated in haemocytes, gill, lymphoid organ and stomach at all time–points post WSSV challenge. Similarly, up–regulated expression of *Pm*TRAF6 was detected in haemocytes and lymphoid organ at late stage of infection. *Pm*Cactus expression was significantly up–regulated in haemocytes at early time–points and lymphoid organ at later time–points tested. *Pm*Dorsal expression was up–regulated in haemocytes and gill, at most of the time–points.

In response to *Vibrio harveyi,* significant up–regulation of these genes was observed in haemocytes, lymphoid organ and gut tissues at different time–points after infection.

*Pm*Toll expression was significantly up–regulated in lymphoid organ, gill, midgut and hindgut, with elevated expression levels. *Pm*Spatzle was significantly up–regulated at all time–points in hepatopancreas and gut tissues. *Pm*MyD88 showed significantly up–regulated with heightened expression levels in all the tissues. Similarly *Pm*IRAK–4 expression was significantly up–regulated in all tissues except hindgut. *Pm*TRAF6 showed up–regulated expression in hepatopancreas as well as hindgut at all the time–points and at most of the time–points in other tissues. *Pm*Cactus expression was up–regulated in gill stomach and hepatopancreas at all the time–points, while expression of *Pm*Dorsal was up–regulated in all tissue at all the time–points except hindgut.

4.4. Immune gene expression *in vitro* in response to WSSV and selected ligands using primary cultures of haemocytes of *Penaeus monodon*

Primary cell cultures were developed from haemocytes, of *P. monodon* The expression analysis of different immune genes, in response to WSSV and on exposure to pathogen–mimicking ligands poly I:C, LPS and peptidoglycan was studied in the primary haemocyte cell culture at different time–points post–exposure (2, 6, 12, 24, 48, 72h). Up–regulation of most of the immune genes was noticed in response to WSSV infection *in vitro*. Upon exposure to WSSV, significantly upregulated expression was observed for *Pm*Toll, *Pm*MyD88, *Pm*TRAF6, and *Pm*Dorsal. *Pm*Spatzle was up–regulated at the early time–points while *Pm*IRAK–4 was up–regulated at all time–points except 2 h post–exposure. *Pm*Cactus was mostly up–regulated at all time–points except 12 and 24h post–exposure. All the immune genes showed modulation of expression upon ligand stimulation. Expression of *Pm*Toll, *Pm*MyD88 and *Pm*Dorsal was significantly up–regulated upon exposure to poly I:C, LPS as well as PGN. *Pm*Spatzle was up–regulated at all time–points except 72 h upon exposure to poly I:C and significantly up–regulated with PGN at all time–points post–exposure. *Pm*IRAK–4 was up–regulated upon exposure to LPS and PGN. While *Pm*TRAF6 and *Pm*Cactus showed down–regulation of expression at most of the time points upon exposure to ligands

SUMMARY

Crustaceans rely on innate immune responses as the first line of defence and, therefore, research on the innate immune system is significant to provide new insights into the management and control of infectious diseases affecting them. The Toll–pathway and its components have been studied in few species of shrimp. In the present study the expression profile of significant immune genes of the Toll–pathway in the giant black tiger shrimp, *Penaeus monodon,* was elucidated to understand the distribution of these genes in immune relevant tissues and their response to invading pathogens (virus–WSSV and bacteria– *Vibrio harveyi*). To investigate the role of Toll–pathway in innate immune response of shrimp, Spatzle (*Pm*Spatzle), MyD88 (*Pm*MyD88), TRAF6 (*Pm*TRAF6), Cactus (*Pm*Cactus), Dorsal (*Pm*Dorsal) were identified and characterised. The expression profiles in response to pathogen challenge and ligand stimulation was also elucidated for previously identified genes – TLR (*Pm*Toll) and Tube (*Pm*IRAK–4).

In the present study–full length sequences of *Pm*Spatzle,*Pm*MyD88, *Pm*TRAF6, as well as partial sequences (including 5' UTR) of *Pm*Cactus and *Pm*Dorsal were generated. Full–length cDNA sequences were obtained using RT–PCR and 3'/5' RACE PCR employing different primers designed based on available sequences in the GenBank. Partial sequences included coding region and 5' UTR. The sequences were analysed using different bioinformatics tools such as BLAST algorithm (NCBI), ORF finder (NCBI), ExPaSy tools, and SMART and compared with the reported sequences. Following sequence analysis, specific domains could be identified which were typical for that particular gene. In *Pm*Spatzle the conserved C–terminal activated 'Spaetzle' domain, in *Pm*MyD88 a TIR domain (residues 157–349 in the deduced amino acid sequence), in *Pm*TRAF6– TRAF homology (MATH) domain (residues 419–543), in *Pm*Cactus six ankyrin repeats (ANKs) distributed in the 175–398 region and in *Pm*Dorsal a Rel homology domain (RHD) in the 82–252 region which is a common feature of the NF–κB family proteins were predicted. Further, multiple sequence alignments of deduced protein sequence and phylogenetic analysis of these genes revealed the homology of these genes with same genes in other shrimp species.

An important aspect of this study was the elucidation of the ontogenetic expression of these immune genes, and the constitutive expression of these genes in the different developmental stages indicate the significance of these genes in the physiological and immunological functions during early development of shrimp. In the tissue–level expression

profile in healthy animals, the selected genes showed constitutive expression in all the fifteen tissues tested with variation in the level of expression among the tissues. The tissue–level expression of *Pm*Toll was found to be the highest in lymphoid organ and the lowest in muscle. In the case of *Pm*MyD88, midgut showed the highest expression and haemocytes showed the highet expression of *Pm*IRAK–4 level. *Pm*Spatzle and *Pm*TRAF6 expression was detected at the highest level in eyestalk. Highest expression of *Pm*Cactus could be noticed in midgut and minimum in cephalic ganglion. *Pm*Dorsal expression was maximum in lymphoid organ and minimum in hepatopancreas.

Previously, many researchers have studied the expression of innate immune genes including the genes in the Toll pathway in response to challenge with either bacteria or virus or a pathogen mimicking ligand. However, in the present study both *in vivo* and *in vitro* based approach was used to elucidate response of these genes to pathogen challenge or to pathogen mimicking ligands. In the *in–vivo* study, healthy, juvenile *P. monodon* were injected intramuscularly with PBS suspension of WSSV (100 µL of 1.3×10^3 copies/µL) or *Vibrio harveyi* (100 µL of 1×10^6 CFU/mL). Un–infected animals were used as zero hour control. The temporal expression of *Pm*Toll, *Pm*Spatzle, *Pm*MyD88, *Pm*IRAK–4, *Pm*TRAF6, *Pm*Cactus, *Pm*Dorsal at different time–points post–challenge was studied in comparison with the control using real time PCR with EF–1α as the internal reference gene. The *in vitro* experiment was carried out on primary culture developed from haemocytes of healthy *P. monodon* . For the ligand–specificity study, the primary culture of haemocytes was exposed to a live virus (WSSV), a viral mimicking substance– poly I:C (50 ng/mL), and two ligands mimicking the bacterial pathogens, lipopolysaccharides (LPS, 10 µg/mL) and peptidoglycan (PGN, 10 µg/ mL); in separate experiments. The expression profile of the genes *in vitro* was elucidated at different time–points post–exposure using real–time PCR. Significant modulation of the expression of these genes upon challenge with WSSV, *Vibrio harveyi* and on exposure to ligands provides the evidence that the Toll pathway in shrimp–*Penaeus monodon* responds to viral and bacterial infection.

In conclusion, the present study is expected to enable a better understanding of the innate immune system and immune genes involved in pathogen recognition as well as the downstream signaling pathways in *Penaeus monodon* by identification of key immune genes of Toll pathway, determining its tissue level and ontogenetic expression and the modulation of expression in response to the most significant viral and bacterial pathogens infecting shrimp. This would also

provide new insights into the role of immune genes and help in developing protection strategies to combat diseases of *P. monodon.* Some of the observations in the study have already been published in peer–reviewed journals (Deepika et al., 2014; Sreedharan et al., 2017; Sreedharan et al., 2018; Deepika et al., 2019; Deepika et al., 2020).

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